

Supplementary Material

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Agents and antibodies

T315 was synthesized in the authors' laboratory [1]. DAPT, doxycycline, and puromycin were purchased from Sigma (St. Louis, MO). Antibodies used in this study and their sources were as follows: Antibodies for ILK (#3856), p-Ser473-Akt (#4060), Akt (#9272), Notch1^{V1754} (#2421), Notch1 (#3608), Notch2 (#4530), Notch3 (#5276), Notch4 (#2423), ABCG2 (#4477), c-Myc (#5605), ZEB1 (#3396), Bmi1 (#6964), YB-1 (#4202), PS1 (#5643), PEN-2 (#8502), NCT (#5665), caveolin-1 (#3267), and GFP (#2955) were purchased from Cell Signaling Technology (Beverly, MA); HES1 (sc-25392), Twist (sc-15393), β -catenin (sc-7963), GAPDH (sc-32233), and integrin β 3 (sc-14009) from Santa Cruz Biotechnology (Santa Cruz, CA); Nestin (611658) from BD Biosciences (San Jose, CA); β -actin (8691002) from MP Biomedicals (Irvine, CA); HRP-conjugated anti-mouse IgG (NC9743863) and anti-rabbit IgG (NC9877945) from ImmunoResearch Laboratories (West Grove, PA); and biotinylated goat anti-rabbit antibody from Vector Laboratories (Burlingame, CA). Notch1^{V1754} antibody recognizes the active form of human Notch1 (Notch1^{NICD}), specifically binding to the exposed cleavage epitope, Val-1754.

Cell culture

The human breast cancer cell lines MCF-7, MDA-MB-468 and MDA-MB-231 were purchased from the American Type Culture Collection (Manassas, VA), which authenticates human cell

lines in their collection using short tandem repeat analysis, and were maintained in RPMI 1640 medium (Life Technologies; Grand Island, NY) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin B. SUM-159 cells were obtained from Asterand (Detroit, MI), and maintained in Ham's F-12 (Life Technologies), supplemented with 5% FBS, insulin (5 µg/ml), and hydrocortisone (1 µg/ml), 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamicin B. The MCF-7^{IL-6} cell line was provided by co-author Nicholas J. Sullivan [2]. The MCF7^{IL-6/TRE-shILK}, MCF7^{TRE-IL-6}, MDA-MB-231^{TRE-shILK}, and MDA-MB-231^{shCtl}, MDA-MB-231^{shILK}, SUM-159^{shCtl}, and SUM-159^{shILK} stable lines were created via lentiviral infection and puromycin selection. Cells were incubated at 37°C in a humidified incubator containing 5% CO₂.

Plasmid constructs

ILK (Clone ID: 3457801) and IL-6 (Clone ID: 3884652) expression cDNA in the pCMV-SPORT6 vector, and shRNAs for IL-6 (Clone ID: V3THS_390096, AGGCAAAGAATCTAGATGC) and ILK (Clone ID: V2THS_48753, CACTCAATAGCCGTAGTGT) in the doxycycline-inducible pTRIPZ vector were purchased from GE Dharmacon (Lafayette, CO). The shRNA lentiviral plasmid against ILK (Clone ID: NM_004517.x-1688s1c1, TCAGAGCTTTGTCACCTTGCCAC) or negative control (SHC001) in the pLKO.1-puro vector were purchased from Sigma. FLAG-ILK expression plasmid was constructed in the pBICEP-2 vector (Sigma) by amplifying ILK full-length cDNA by PCR. The doxycycline-inducible IL-6 expression plasmid was constructed in the pLenti CMV_{TRE}3G Puro DEST (w811-1) vector (Addgene #27565) by LR reaction after subcloning IL-6 cDNA into the pENTR noccDB (w48-1) entry vector (Addgene #17398). pLenti CMV_{TRE}3G Puro DEST

(w811-1) vector and pENTR1A no ccDB (w48-1) vector were a gift from Eric Campeau. The GFP-C99 expression plasmid was constructed in the pEGFP-N1 vector (Clontech Laboratories; Mountain View, CA) by amplifying a portion of the coding region of the β -amyloid precursor protein corresponding to amino acids 597–695 from pCAX APP C99 (Addgene #30146) by PCR. pCAX APP C99 plasmid was a gift from Dennis Selkoe and Tracy Young-Pearse. Caveolin-1 siRNA (SR300603B, rGrCrArGrUrUrGrUrArCrCrArUrGrCrArUrUrArArGrArGCT; SR300603C, rCrCrUrArArArCrArCrCrUrCrArArCrGrArUrGrArCrGrUGG) was purchased from OriGene (Rockville, MD).

Lentivirus preparation and infection of breast cancer cell lines

Lentiviral plasmids were cotransfected with Addgene 3rd Generation Packaging Systems, from which pMDLg/pRRE [#12251], pRSV-Rev [#12253] and pMD2.G [#12259] were kind gifts from Didier Trono [3], in 293T cells according to a standard calcium phosphate transfection procedure described by Addgene. The viral particles were collected for infection of target cell lines. The stable cells were selected by exposure to puromycin (0.5-2 μ g/ml) and G418 (250 μ g/ml) for one week.

RNA isolation and real-time quantitative PCR (qPCR)

Cells were washed once with PBS and total RNA was isolated with TRIzol (Thermo Fisher Scientific) and reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad; Hercules, CA). Real-time qPCR was performed on an CFX Connect™ Real-Time PCR Detection System (Bio-Rad) using SsoAdvanced™ SYBR® Green Supermix (Bio-Rad) with the

following primers to amplify the corresponding mRNA of human origin:

PS1, 5'-GCGGCGGGGAAGCGTATACC-3'; 5'-GGCCAAGCTGTCTAAGGACCGC-3'

NCT, 5'-GGAGAACCAGCCGAATTG-3'; 5'-GGAGTAAACACCAAACCCA-3'

PEN-2, 5'-CCTGCCTTTTCTCTGGTTGGTC-3'; 5'-TCCAGGAGGTGAGCACTATCAC-3'

18S, 5'-ACCCGTTGAACCCCATTCGTGA-3'; 5'-GCCTCACTAAACCATCCAATCGG-3'

Immunoblot analysis

Growing cells were harvested by scraping and lysed in the presence of RIPA lysis buffer containing protease inhibitor cocktail. An equal amount of protein with SDS sample buffer (60 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 5% 2-mercaptoethanol) from each sample was loaded per lane, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane, and proteins were probed with specific antibodies. Secondary antibodies conjugated to HRP and Western Lighting Chemiluminescence Reagent Plus (Perkin-Elmer, Waltham, MA) were used to develop images.

Lipid-raft isolation and dot blotting

Based on instructions accompanying the FOCUS™ Signal Protein Isolation kit (G Biosciences, St. Louis, MO), cells were washed twice with PBS, harvested by scraping, and then lysed by sonication in iced Signal Protein Extraction (SPE) buffer-I supplemented with protease inhibitor cocktail (Sigma). Then, SPE buffer-II was added followed by incubation on ice for 15 min with vigorous mixing (Vortex) for 1 min every 5 min. The lysates were centrifuged at 20000 × g for 15 min at 4°C and supernatants were collected as non-lipid raft fractions. The pellets containing lipid raft protein were solubilized in Focus Protein Solubilization buffer supplemented with

protease inhibitor cocktail (Sigma) by incubation at room temperature for 15 min with vigorous mixing for 1 min every 5 min. The lysates were centrifuged at $18000 \times g$ for 10 min at room temperature and the supernatants were collected as lipid raft fractions. The protein concentration of each sample was quantified and adjusted to equal amounts of protein with SDS sample buffer for immunoblotting.

Immunofluorescence analysis

Nuclei were stained with DAPI contained in the Vectashield mounting medium (Vector Laboratories). Images were obtained with an Olympus FV1000 Filter Confocal Microscope (Olympus Corp., Japan) with optics field (40 \times or 60 \times) using FV300/FV500/FV1000 Version 5 application software (FV10-SW). For merged images, pseudocolor presentation shows Alexa 647-conjugated cholera toxin in orange, Alexa 488-conjugated marker in green, Alexa 555-conjugated marker in red, and DAPI in blue.

Reporter assays

The Notch transactivation activities were measured using the Signal Reporter Assay Kits (CCS-014L, Qiagen, Valencia, CA) according to the manufacturer's instructions. The reporter plasmids were transfected by Lipofectamine 2000 (Life Technologies), and the Dual-Luciferase Reporter Assay System (Promega) was used for detecting luciferase luminescence with a Promega GloMax® 96 microplate luminometer.

ALDEFLUOR assays

Based on instructions accompanying the ALDEFLUOR kit (StemCell Technologies), treated

cells were trypsinized, harvested, and suspended in buffer containing ALDH substrate (BAAA 1 mmol per 1×10^6 cells) and incubated at 37°C for 30 min. In each experiment, the specific ALDH inhibitor, diethylaminobenzaldehyde (DEAB), (50 mmol/L) was used as negative control.

Animal studies

Female NOD/SCID mice (NOD.CB17-*Prkdc*^{scid}/NCrHsd; 5-6 weeks of age; Harlan, Indianapolis, IN) were group-housed under conditions of constant photoperiod (12 hours light: 12 hours dark) with *ad libitum* access to sterilized food and water. All experimental procedures using mice were done in accordance with protocols approved by The Ohio State University Institutional Animal Care and Use Committee. All xenograft tumors were established by injection of cancer cells into mammary fat pads of female NOD/SCID mice. For all injections, cells were suspended in Matrigel (BD Biosciences, San Jose, CA). Tumors were measured up to twice weekly with calipers and the volumes were calculated using $V = 1/2 (\text{width}^2 \times \text{length})$.

In the experiment to assess the effect of ILK inhibition by treatment with T315 on CSC populations, T315 was prepared for oral administration in a vehicle containing 10% DMSO, 0.5% methylcellulose, and 0.1% Tween 80 in sterile water. At the end of drug treatment, the mice were humanely euthanized and tumors were harvested and dissociated for evaluation of the CSC subpopulation by ALDEFLUOR assay, mammosphere formation, and tumorigenicity after re-implantation into secondary recipient NOD/SCID mice. Tumor dissociation and tumor cell re-implantation are described below.

Tumor dissociation

Tumors harvested from T315- and vehicle-treated mice were minced by scalpel and incubated in

medium 199 (Life Technologies) containing collagenase/hyaluronidase (StemCell Technologies) at 37°C for 30 minutes. The tissues were further dissociated by pipetting and then passed through a 40-µm nylon mesh (BD Pharmingen) to produce a single-cell suspension. Viable human cells were sorted from non-viable and mouse cells by FACS. Before the ALDEFLUOR reaction was performed, cells were stained with an anti-H2Kd antibody conjugated with Alexa Fluor® 647 (mouse MHC I, BD Pharmingen) at 1/250 dilution for 30 min on ice for exclusion of mouse cells. After the ALDEFLUOR reaction, cells were incubated with 7-aminoactinomycin D (7-AAD) (BD Pharmingen) for 5 min on ice for exclusion of non-viable cells. The sorting gates were established based on negative controls for viability (cells stained with 7-AAD only) and for human cells (cells stained with anti-H2Kd antibody only). The gating for the ALDH^{br} population established based on DEAB negative controls.

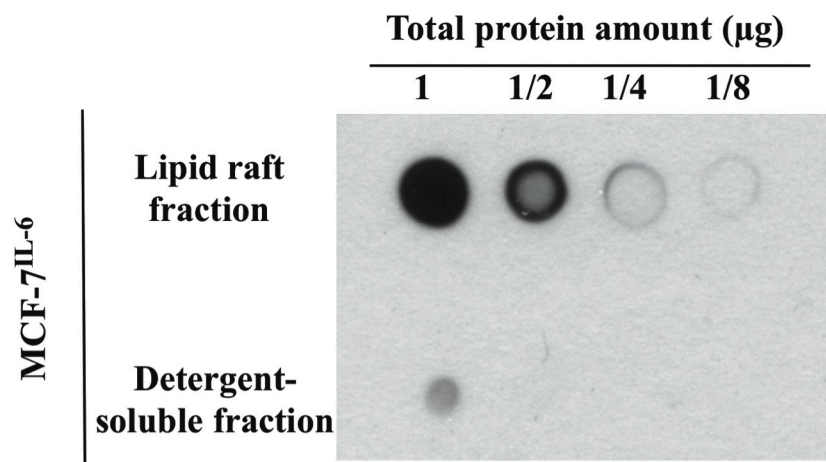
Tumor initiation after re-implantation into secondary NOD/SCID mice

Viable SUM-159 cells isolated from tumors from T315-treated and vehicle-treated mice as described above were injected into the inguinal mammary fat pads of naïve secondary NOD/SCID mice (n = 8). Each mouse was inoculated with 1000 cells from control mouse tumors in the left inguinal mammary fat pad and another 1000 cells from T315-treated mouse tumors in the contralateral mammary fat pad. The emergence of tumors was monitored twice weekly by palpation for up to 49 days post-injection.

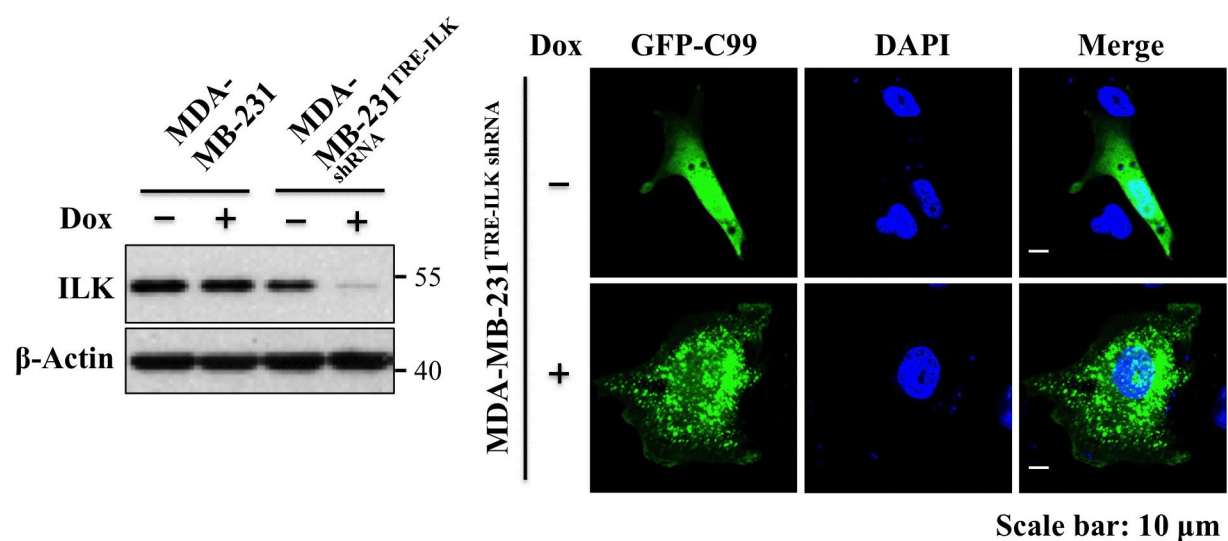
References

- [1] Lee SL, Hsu EC, Chou CC, Chuang HC, Bai LY, Kulp SK, et al. Identification and characterization of a novel integrin-linked kinase inhibitor. *J Med Chem.* 2011;54:6364-74.
- [2] Sasser AK, Sullivan NJ, Studebaker AW, Hendey LF, Axel AE, Hall BM. Interleukin-6 is a potent growth factor for ER-alpha-positive human breast cancer. *FASEB J.* 2007;21:3763-70.
- [3] Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, et al. A third-generation lentivirus vector with a conditional packaging system. *J Virol.* 1998;72: 8463-71.

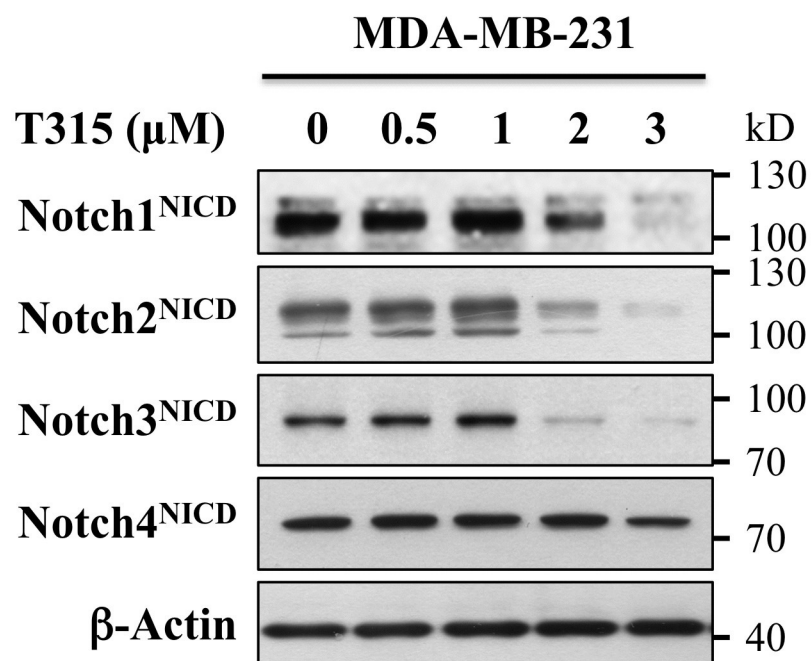
2. Supplementary Figures



Supplementary Figure S1. Confirmation of lipid raft isolation. Dot blot using HRP-conjugated cholera toxin to detect the lipid raft marker ganglioside GM1 in the lipid raft versus detergent-soluble fractions isolated from MCF-7^{IL-6} cells.

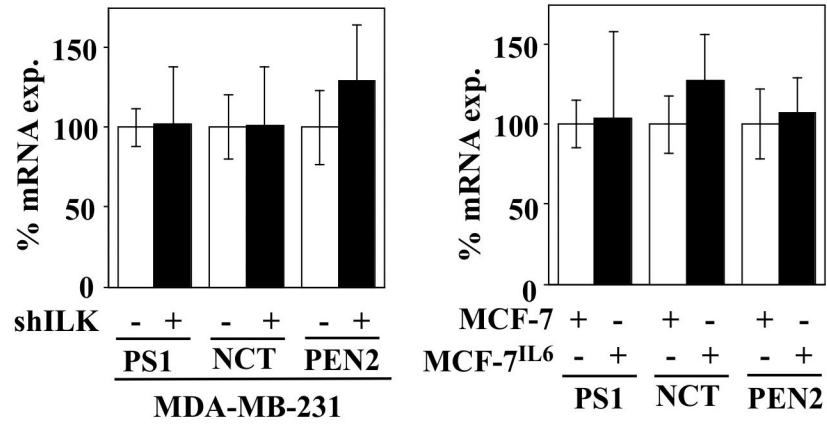


Supplementary Figure S2. ILK knockdown suppressed γ -secretase activity. *Left*, Doxycycline (Dox)-inducible shRNA-mediated knockdown of ILK in MDA-MB-231^{TRE-shILK} cells. *Right*, Immunocytochemical analysis of the cellular distribution of the GFP-labelled γ -secretase substrate C99 (GFP-C99) in GFP-C99-expressing MDA-MB-231^{TRE-shILK} cells treated with doxycycline or vehicle. The change in distribution indicates the inhibition of γ -secretase activity in cells with suppressed ILK expression.



Supplementary Figure S3. Inhibitory effect of T315 on the activation of Notch isoforms.

Western blot analysis of the dose-dependent effects of T315 on the activation status of Notch isoforms 1-4 in MDA-MB-231 cells.



Supplementary Figure S4. Neither IL-6, nor ILK affects mRNA expression of PS1, NCT and PEN-2. The effects of ILK knockdown in MDA-MB-231 cells (*left*) or IL-6 over-expression in MCF-7 cells (*right*) on the mRNA expression of γ -secretase components (PS1^{CTF}, NCT, and PEN-2) were assessed by qPCR.